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ORIGINAL ARTICLE

Prostaglandin E_2 (PGE_2) downregulates interleukin (IL)-1 α-induced IL-6 production via EP_2/EP_4 subtypes of PGE_2 receptors in human periodontal ligament cells

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OBJECTIVES: Prostaglandin E_2 (PGE_2) exerts its biological actions via EP receptors, which are divided into four subtypes, EP_1, EP_2, EP_3 and EP_4. In the present study, we examined whether PGE_2 regulated interleukin (IL)-1α-induced IL-6 production in human periodontal ligament (PDL) cells and if so, which subtypes of PGE_2 receptors were involved.

METHODS: PDL cells were stimulated with vehicle or IL-1α in the presence or absence of indomethacin (a cyloxygenase inhibitor), PGE_2 or various EP agonists. IL-6 and PGE_2 levels were measured by enzyme-linked immunosorbent assay. EP receptor mRNA expression was examined by reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Indomethacin significantly enhanced IL-1α-induced IL-6 production by PDL cells, although it completely inhibited IL-1α-induced PGE_2 production. Exogenous PGE_2 significantly suppressed IL-1α-induced IL-6 production. Butaprost, a selective EP_2 agonist, and ONO-AE1-329, a selective EP_4 agonist, significantly inhibited IL-1α-induced IL-6 production, although 17-phenyl-ω-trinor PGE_2, an EP_1 agonist, and ONO-AP-324, an EP_3 agonist, did not affect it. RT-PCR analysis showed that EP_2 and EP_4 mRNA was expressed in PDL cells.

CONCLUSIONS: We suggest that PGE_2 downregulates IL-1α-induced IL-6 production via EP_2/EP_4 receptors in human PDL cells.

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Keywords: EP receptor; prostaglandin E_2; interleukin-6; periodontal ligament cells

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Introduction

Periodontal disease is characterized by gingival inflammation and loss of periodontal attachment apparatus, frequently causing tooth loss. Prostaglandins (PGs) including PGE_2 are thought to be involved in the pathogenesis of periodontal disease (Offenbacher *et al*, 1993). It has been demonstrated that non-steroidal anti-inflammatory drugs, which inhibit PG synthesis, are effective to prevent periodontal destruction (Williams *et al*, 1989). PGE_2 levels are reportedly enhanced in inflamed human gingiva, compared with normal gingiva (Ohm *et al*, 1984). Furthermore, PGE_2 has been shown to be a potent stimulator of bone resorption (Klein and Raisz, 1970) and to be associated with attachment loss (Offenbacher *et al*, 1984, 1993).

Recent studies have demonstrated that the cellular effects of PGE_2 are mediated by activation of multiple functionally distinct subtypes of PGE_2 receptors, which are classified into EP_1, EP_2, EP_3 and EP_4, based on their ligand binding selectivities and activation mechanisms of signaling pathway (Negishi *et al*, 1995; Narumiya *et al*, 1999). EP_1 receptors mediate increase in intracellular calcium levels. EP_2 and EP_4 receptors activate adenylate cyclases via a cholera toxin-sensitive, stimulatory G protein and elevate intracellular cAMP levels. EP_2 receptors are sensitive to butaprost, an agent that selectively binds PGE_2 receptors, whereas EP_4 receptors are not. Multiple isoforms of EP_3 receptors with different C-terminal tails are generated by alternative mRNA splicing. EP_3 receptor variants mediate several signaling pathways including inhibition and stimulation of adenylate cyclase, activation of phospholipase C and mobilization of intracellular calcium.

Interleukin (IL)-6 is a pleiotropic cytokine produced by a variety of cells including monocytes/macrophages, activated T cells, endothelial cells and fibroblasts (Van Snick, 1990). IL-6 promotes B-cell activation and induces hepatocytes to produce acute phase proteins (Van Snick, 1990). Furthermore, IL-6 stimulates induction of osteoclast formation and bone resorption (Ishimi *et al*, 1990). It has been reported that IL-6 levels in gingival crevicular fluid are correlated with bleeding

index and probing depth in patients with adult periodontitis (Geivelis *et al*, 1993). The periodontal ligament (PDL) is the unique soft tissue which binds the teeth to the alveolar bone proper. PDL cells are a heterogenous cell population including fibroblastic and mineralized tissue-forming cells (Beersten *et al*, 1997). *In vitro* PDL cells have been shown to synthesize IL-6 in response to stimuli including IL-1α (Agarwal *et al*, 1995). Previously, we demonstrated that PDL cells produce high levels of PGE_2 in response to IL-1α (Noguchi *et al*, 1999). Several studies have demonstrated that IL-6 generation is regulated by PGE_2 in several types of cells including osteoblasts, astrocytoma cells and gingival fibroblasts (Blom *et al*, 1997; Takaoka *et al*, 1999; Noguchi *et al*, 2002). Thus, it is very likely that PGE_2 regulates IL-1α-induced IL-6 production in PDL cells.

In the present study, we investigated the effect of PGE_2 on IL-1α-induced IL-6 production in PDL cells derived from periodontally healthy subjects and examined which subtypes of PGE_2 receptors were involved.

Materials and methods

Cell preparation

Human PDL cells were obtained from explants of PDLs from premolars or third molars extracted from three periodontally healthy subjects. Our study protocol, including biopsy of gingival tissue, satisfied the ethical standards of Tokyo Medical and Dental University and informed consent was obtained from all the subjects. The cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) (Bioserum, Victoria, Australia) in the presence of 100 U ml^{-1} of penicillin (Sigma Chemical Co., St Louis, MO, USA) and 100 $\mu g\ ml^{-1}$ of streptomycin (Sigma Chemical Co.) in a humidified atmosphere of 5% CO_2 at 37°C. The cells used for experiments were between the fifth and tenth passages.

Cell stimulation

PDL cells were seeded into 96-well plates at a concentration of 5×10^4 cells ml^{-1}. When the cells reached confluence, the medium was changed to α-MEM containing 0.5% FBS to minimize any serum-induced effects on PDL cells. After 24 h, the cells were stimulated with vehicle or 2 ng ml^{-1} of human recombinant IL-1α (Sigma Chemical Co.) in the presence or absence of indomethacin (Wako, Tokyo, Japan), PGE_2 (Cascade Biochem Ltd, Berkshire, UK), 17-phenyl-ω-trinor PGE_2 (Cayman Chemical, Ann Arbor, MI, USA), butaprost (a gift from ONO Pharmaceutical Co. Ltd, Tokyo, Japan), ONO-AP-324 (a gift from ONO Pharmaceutical Co. Ltd), ONO-AE1-329 (a gift from ONO Pharmaceutical Co. Ltd), dibutyryl cAMP (Wako) and forskolin (Wako) in the indicated combination and concentrations, which were added to the cells 30 min prior to stimulation with IL-1α.

PGE_2 and IL-6 assay

The levels of PGE_2 and IL-6 in the conditioned media collected from control or IL-1α-stimulated cells were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (PGE_2, Amersham Pharmacia Biotech, Buckinghamshire, UK; IL-6, Endogen Inc., Woburn, MA, USA).

Reverse transcription-polymerase chain reaction

Total cellular RNA was isolated from confluent PDL cells. In brief, the cells were lysed and RNA was extracted with the guanidinium thiocyanate/phenol/chloroform method, using ISOGEN (Nippon gene, Toyama, Japan). After extracting total RNA from PDL cells, cDNAs were synthesized from 2 μg of total RNA with RAV2 reverse transcripatase and oligo(dT)primers (Takara Co., Shiga, Japan), as described previously. The specific primer pairs for human EP_2, EP_4 and β-actin were selected, according to the cDNA sequences reported by Funk *et al* (1993), Bastien *et al* (1994), Regan *et al* (1994) and Ponte *et al* (1984), respectively. The primers were EP_1: sense primer, 5′-TCTACCTCCCTGCAGCGGCCACTG-3′, antisense primer, 5′-GAAGTGGCTGAGGCCGCTGTGCCGGGA-3′; EP_2: sense primer, 5′-TTCATCCGGCACGGGCGGACCGC-3′, antisense primer, 5′-GTCAGCCTGTTTACTGGCATCTG-3′; EP_4: sense primer, 5′-CCTCCTGAGAAAGACAGTGTC-3′, antisense primer, 5′-AGGACTCAGAGAGTGTCTT-3′; and β-actin: sense primer, 5′-GTGGGCATGGGTCATCAGAAGGAT-3′, antisense primer, 5′-CTCCTTAATGTCACGCACGATTTC-3′. The PCR reaction was performed using 25 μM of each primer, 25 mM of each dNTP and 2.5 units of Taq DNA polymerase (Takara Co.) in an automated DNA thermal cycler (Takara Co.). The PCR amplification was comprised of 35 cycles (EP_2 and EP_4) or 25 cycles (β-actin) of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 3 min. The reverse transcription-polymerase chain reaction (RT-PCR) products were resolved by electrophoresis in 2% agarose gels and stained with ethidium bromide. The identity of the RT-PCR products of EP_2 and EP_4 was confirmed by restriction endonuclease digestion and nucleotide sequence analysis (data not shown).

Statistical analysis

Data are expressed as the mean ± standard deviation (s.d.). Data were subjected to one-way analysis of variance (ANOVA), using the StatView 4.0 program on a Macintosh computer. Fisher's protected least significance test was used in the *post hoc* comparison of specific groups.

Results

Effect of indomethacin on IL-1α-induced IL-6 and PGE_2 production in PDL cells

To examine whether PGs endogenously produced affected IL-6 production in IL-1α-stimulated PDL cells, the effect of indomethacin, a cyclooxygenase inhibitor, on IL-6 production was investigated. Indomethacin significantly increased IL-1α-induced-IL-6 production ($P < 0.01$), although PGE_2 production by

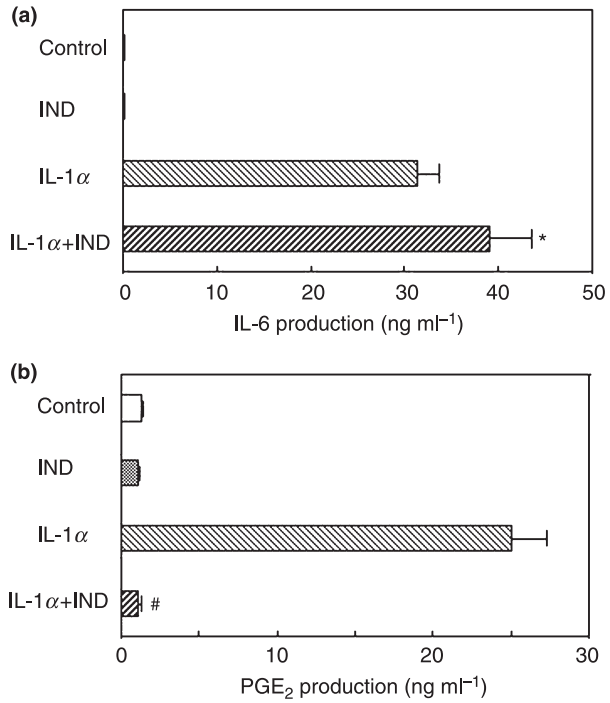

Figure 1 Effect of indomethacin on production of IL-6 (**a**) and PGE_2 (**b**) in IL-1α-stimulated PDL fibroblasts. PDL fibroblasts were stimulated with vehicle or 2 ng ml^{-1} of IL-1α in the presence or absence of 1 μM of indomethacin (IND) for 24 h. After incubation, IL-6 levels (**a**) and PGE_2 levels (**b**) in the culture media were measured by ELISA, as described in Materials and methods. Values are mean ± s.d. ($n = 4$). *Significantly different from control ($P < 0.01$). # Significantly different from IL-1α ($P < 0.01$)

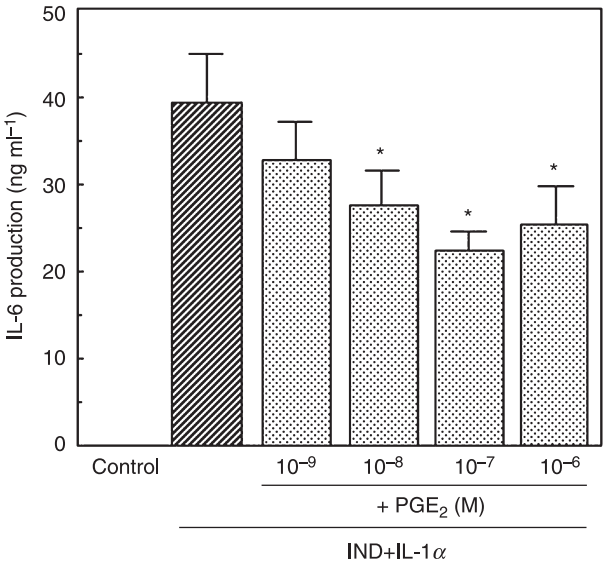

Figure 2 Effect of PGE_2 on IL-6 production in IL-1α-stimulated PDL cells. PDL cells were treated with vehicle or 2 ng ml^{-1} of IL-1α in the presence of 1 μM of indomethacin (IND) and various doses of PGE_2 for 24 h. After incubation, IL-6 levels in the culture media were measured by ELISA, as described in Materials and methods. Values are mean ± s.d. ($n = 4$). *Significantly different from IND + IL-1α ($P < 0.01$)

IL-1α was completely inhibited by indomethacin (Figure 1a,b), which suggests that endogenous PGs were involved in downregulation of IL-1α-induced IL-6 production.

Effect of PGE_2 and EP agonists on IL-1α-induced IL-6 production in PDL cells

The effect of exogenous PGE_2 and EP agonists on IL-6 generation was investigated. PGE_2 alone induced an increase of IL-6 production in a dose-dependent manner. However, the levels of PGE_2-induced IL-6 were approximately 50-fold less, compared with the levels of IL-1α-induced IL-6. Next, we examined the effect of PGE_2 and EP agonists on IL-1α-induced IL-6 production. PDL cells were treated with indomethacin to exclude the involvement of endogenous PGE_2 prior to addition of PGE_2 and EP agonists. Exogenous PGE_2 suppressed IL-1α-induced IL-6 production (Figure 2). Butaprost, a selective EP_2 agonist, and ONO-AE1-329, a selective EP_4 agonist, reduced IL-1α-induced IL-6 production in a concentration-dependent fashion (Figure 3). 17-Phenyl-ω-trinor PGE_2, an EP_1 agonist, tended to increase IL-1α-induced IL-6 production, although not significant, and ONO-AP-324, a selective EP_3 agonist, had no effect on IL-1α-induced IL-6 production (data not shown). These data suggest that

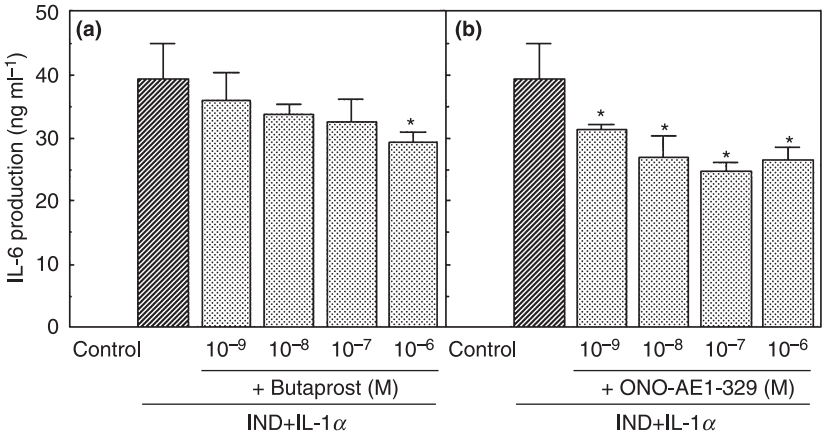

Figure 3 Effect of butaprost and ONO-AE1-329 on IL-6 production in IL-1α-stimulated PDL cells. PDL cells were treated with vehicle or 2 ng ml^{-1} of IL-1α in the presence of 1 μM of indomethacin (IND) with or without various doses of butaprost or ONO-AE1-329 for 24 h. After incubation, IL-6 levels in the culture media were measured by ELISA, as described in Materials and methods. Values are mean ± s.d. ($n = 4$). *Significantly different from IL-1α + IND ($P < 0.01$)

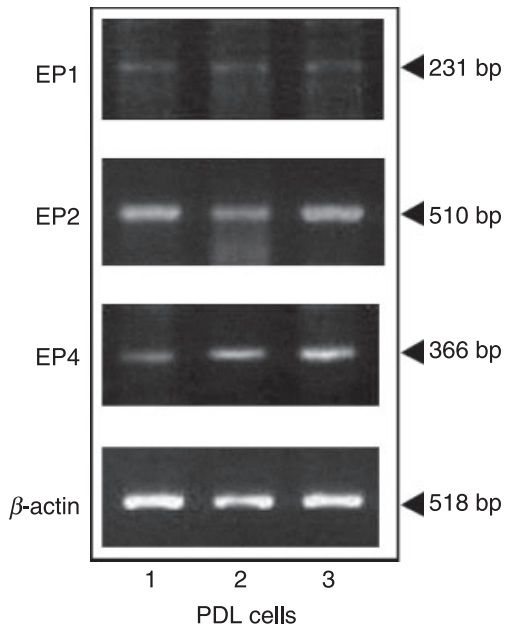

Figure 4 Detection of mRNA expression of EP_1, EP_2 and EP_4 receptors. Total RNA was extracted from PDL cells of three subjects and RT-PCR was performed to detect mRNA expression of EP_1, EP_2 and EP_4 receptors

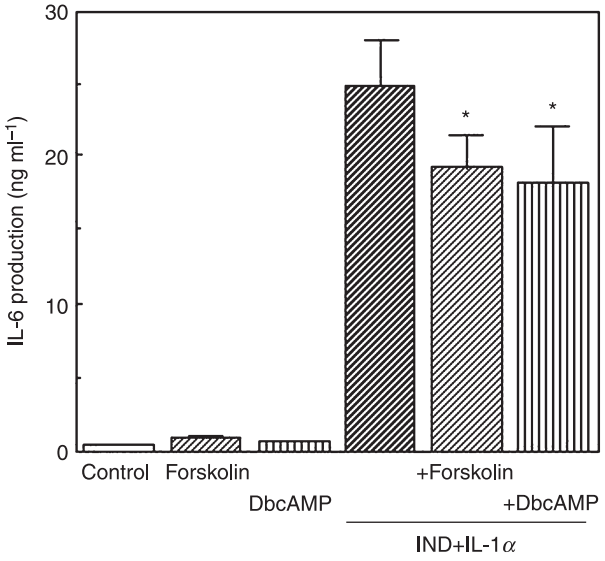

Figure 5 Effect of forskolin and dibutyryl cAMP on IL-6 production in IL-1α-stimulated PDL cells. PDL cells were treated with vehicle or 2 ng ml^{-1} of IL-1α in the presence of 1 μM of indomethacin (IND) and 10 μM of forskolin and 100 μM of dibutyryl cAMP (dbcAMP). After incubation, IL-6 levels in the culture media were measured by enzyme-linked immunosorbent assay, as described in Materials and methods. Values are mean ± s.d. ($n = 4$). *Significantly different from IND + IL-1α ($P < 0.05$)

EP_2 and/or EP_4 receptors were involved in PGE_2 downregulation of IL-1α-induced IL-6 production.

Expression of EP_1, EP_2 and EP_4 mRNA in PDL cells
We examined mRNA expression of EP_1, EP_2 and EP_4 receptors in PDL cells by RT-PCR. As shown in Figure 4, EP_1, EP_2 and EP_4 mRNA was expressed in all three samples of PDL cells.

Effect of cAMP-elevating agents on IL-1α-induced IL-6 production
We examined the effect of intracellular cAMP on IL-1α-induced IL-6 production. Dibutyryl cAMP, a cAMP analog, and forskolin, an activator of adenylate cyclases, reduced IL-6 production by IL-1α-stimulated PDL cells (Figure 5).

Discussion

In the present study, we demonstrated that PGE_2 suppressed IL-1α-induced IL-6 production in PDL cells derived from periodontally healthy subjects. Treatment of IL-1α-stimulated PDL cells with indomethacin, a cyclooxygenase inhibitor, significantly enhanced IL-6 production, although IL-1α-induced PGE_2 production was completely inhibited (Figure 1a,b). This finding is consistent with the report by Okada *et al* (1997). Furthermore, exogenous PGE_2 attenuated IL-1α-induced IL-6 production in a dose-dependent manner (Figure 2). These data suggest that PGE_2 endogenously produced is involved in regulation of IL-6 production in IL-1α-stimulated PDL cells.

The diversity and selectivity of the effects of PGE_2 are dependent on expression of EP subtypes of PGE_2 receptors. EP_1 receptors and EP_2/EP_4 receptors are involved in increases in intracellular calcium levels and elevation of intracellular cAMP levels, respectively (Negishi *et al*, 1995; Narumiya *et al*, 1999). It is unclear what types of EP receptors are expressed in PDL cells. Butaprost, a selective EP_2 agonist, and ONO-AP1-329, an EP_4 agonist, inhibited IL-6 production by IL-1α-stimulated PDL cells (Figure 3). 17-Phenyl-ω-trinor PGE_2, an EP_1 agonist, tended to increase IL-1α-induced IL-6 production, although not significant, and ONO-AP-324, a selective EP_3 agonist, had no effect on IL-1α-induced IL-6 production (data not shown). RT-PCR analysis showed that mRNA of EP_1, EP_2 and EP_4 receptors was expressed in PDL cells (Figure 4). Furthermore, both EP_2 and EP_4 agonists increased intracellular cAMP levels in PDL cell (data not shown). It has been demonstrated that the actions of PGE_2 in PDL cells are mediated by intracellular cAMP (Nohutcu *et al*, 1993). From these data, we suggest that EP_2 and/or EP_4 receptors are involved in PGE_2 inhibition of IL-1α-induced IL-6 production. It has been shown that PGE_2 inhibits IL-1β-induced IL-6 production via EP_2 and EP_4 receptors in rat synovial cells (Kurihara *et al*, 2001).

cAMP-elevating agents including dibutyryl cAMP and forskolin caused suppression of IL-1α-induced IL-6 production (Figure 3). Therefore, it is very likely that cAMP-dependent pathways via EP_2/EP_4 receptors are involved in PGE_2 downregulation of IL-1α-induced IL-6 production in PDL cells. In the present study, the mechanism by which cAMP attenuates IL-1α-induced IL-6 production in PDL cells was not investigated.

Transcriptional factors including nuclear factor (NF)-IL-6, NF-κB and AP-1 are required for IL-6 gene activation (Dendorfer *et al*, 1994) and cAMP inhibits NF-κB-mediated transcription in human monocytic cells and endothelial cells (Ollivier *et al*, 1996). Zitnik *et al* (1993) have showed that in human lung fibroblasts cAMP downregulates IL-1α-induced IL-6 production, which is mediated by decreased IL-6 gene expression. However, Takigawa *et al* (1994) have reported that IL-1β-induced IL-6 production in HGF may be inhibited by PGE_2 at the post-transcriptional level. Further studies are necessary to reveal the mechanism by which cAMP downregulates IL-1α-induced IL-6 production in PDL cells.

The roles of IL-6 produced by PDL cells remain unclear. IL-6 promotes B-cell activation and induces hepatocytes to produce acute phase proteins (Van Snick, 1990). Furthermore, IL-6 stimulates induction of osteoclast formation and bone resorption (Ishimi *et al*, 1990). It has been reported that IL-6 levels in gingival crevicular fluid are correlated with bleeding index and probing depth in patients with adult periodontitis (Geivelis *et al*, 1993). Therefore, it is likely that IL-6 plays an important role in controlling inflammatory and immune responses in periodontal disease. It has been shown that IL-1β-induced IL-6 production is regulated by PGE_2 in HGF (Agarwal *et al*, 1995; Noguchi *et al*, 2002). Taken together with our present data that PGE_2 inhibited IL-1α-induced IL-6 production in PDL cells, it is plausible that PGE_2 is involved in controlling IL-6 production in periodontally diseased tissues. Further investigation is needed to elucidate the roles of PGE_2 in periodontal disease.

In conclusion, we suggest that PGE_2 downregulates IL-1α-elicited IL-6 production through cAMP-dependent pathways via EP_2/EP_4 receptors in human PDL cells. Expression and function of EP_2 and EP_4 receptors in PDL cells may play critical roles in controlling inflammatory periodontal conditions.

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